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# Molecular differences in *IDH* wildtype glioblastoma according to *MGMT* promoter methylation

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**Running title:** Molecular differences according to *MGMT* methylation

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## Abstract

**Background:** *O6-methylguanine-DNA-methyltransferase (MGMT)* promoter methylation status is a predictive biomarker in glioblastoma. We investigated whether this marker furthermore defines a molecularly distinct tumor subtype with clinically different outcome.

**Methods:** We analyzed copy number alteration (CNV) and methylation profiles of 1095 primary and 92 progressive *Isocitrate dehydrogenase* (*IDH*) wildtype glioblastomas, including paired samples from 49 patients. DNA mutation data from 182 glioblastoma samples of The Cancer Genome Atlas (TCGA) and RNA expression from 107 TCGA and 55 Chinese Glioma Genome Atlas samples were analyzed.

**Results:** Among untreated glioblastomas, *MGMT* promoter methylated (*mMGMT)* and unmethylated (*uMGMT)* tumors did not show different CNV or specific gene mutations, but a higher mutation count in *mMGMT* tumors. We identified three methylation clusters. Cluster 1 showed the highest average methylation and was enriched for *mMGMT* tumors. Seventeen genes including *GBX2* were found to be hypermethylated and downregulated on mRNA level in *mMGMT* tumors.

In progressive glioblastomas, *PDGFRA* and *GLI2* amplifications were enriched in *mMGMT* tumors. *mMGMT* tumors gain *PDGFRA* amplification whereas *uMGMT* tumors with *PDGFRA* amplifications frequently lose this amplification upon progression. Glioblastoma patients surviving < 6 months and *mMGMT* harbored less frequent *EGFR* amplifications, more frequent *TP53* mutations and a higher TNF-NFκB pathway activation compared to patients surviving longer than 12 months.

**Conclusions:** *MGMT* promoter methylation status does not define a molecularly distinct glioblastoma subpopulation among untreated tumors. Progressive *mMGMT* glioblastomas and *mMGMT* tumors of patients with short survival tend to have more unfavorable molecular profiles.

**Keywords**

$O^6$-methylguanin-DNA methyltransferase (MGMT), glioblastoma biomarker, TERT, NFκB, PDGFRA

## Importance of the Study

This study compares more than 1200 glioblastomas from three sources for differences in methylation, CNV, mutation and RNA expression according to their *MGMT* promoter methylation status. There was an uneven distribution of *MGMT* promoter methylation in three defined methylation clusters, despite otherwise similar molecular profiles. The study is of major relevance for upcoming clinical trials that will stratify and include patients with newly diagnosed glioblastoma according to their *MGMT* promoter methylation status and frequently withhold temozolomide in on the experimental study arms. Differential analysis of primary and progressive tumors revealed differences upon progression including activation of the TNF-NFKB pathway and CNV changes in *mMGMT* tumors that are distinct from *uMGMT* tumors.

## Introduction

*O6-methylguanine-DNAmethyltransferase (MGMT)* promoter methylation status has been consistently identified and used as predictive biomarker for response to alkylating chemotherapy in patients with glioblastoma[1-3]. MGMT is a protein that repairs damage induced by alkylating chemotherapies including temozolomide. Methylation of the *MGMT* promoter leads to a reduced *MGMT* expression[4]. Especially patients with wildtype *isocitrate dehydrogenase 1/2 (IDH)* genes and an unmethylated *methylguanine-O6-methytransferase (MGMT)* promoter show minimal response to chemotherapy with a median survival of just over one year when treated with radiochemotherapy after maximal safe resection[5].

The concept of trials with replacement of temozolomide in the experimental arm in favor of a combination of an experimental systemic therapy with radiotherapy has been successfully established[6-9]. Preclinical data do not suggest an impact of MGMT on radiotherapy, but other treatments have not been systematically evaluated[10]. NOA-04 provided a clinical basis for *MGMT* promoter methylation status being predictive also in *IDH* wildtype anaplastic gliomas[11,12], as did NOA-08 for elderly patients with glioblastoma[13].

In the present study, we aimed to evaluate the molecular differences between glioblastomas with a methylated or unmethylated *MGMT* promoter in different datasets.

## Methods

*Glioblastoma patient cohorts*

As of August 2nd, 2016, we screened the Heidelberg 450k methylation array database. We identified 1028 glioblastoma samples at diagnosis and 66 glioblastoma samples at tumor relapse (see Supplementary Methods) with *IDH* wild-type. *MGMT* promoter methylation status was determined by the algorithm of Bady et al.[14] Samples were classified as 'unsure' and excluded from the analysis if the confidence interval of the prediction included the cutoff value of 0.358. Patients provided informed consent concerning the use of their tissue samples for research purpose. The study was approved by the local ethics committee (No. 206/2005). Of the 1028 primary tumors, 143 tumors with clinical trial grade clinical follow up data and treatment according to the present standard of care were accessible for survival analysis.

*Illumina 450k array platform analysis*

The Illumina Infinium HumanMethylation450 (450k) array was used to obtain the DNA methylation status at 482,421 CpG sites (Illumina, San Diego, CA, USA), at the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ) (see Supplementary Methods)[15].

*Copy number variation (CNV) analysis*

Copy numbers of single genes were assessed from 450k array data (Details in the Supplement).

*The Cancer Genome Atlas (TCGA) data analysis*

TCGA data of 261 glioblastoma specimens at diagnosis were downloaded from the cBio portal (www.cbioportal.org)[17,18] and from firebrowse (Broad institute, Cambridge, MA, firebrose.org)[19] on September 12th 2016 (table S1). Only tumor samples with *IDH 1/2* wildtype and defined *MGMT* promoter methylation status were used for analysis. Clinical data, CNVs, mutation and expression subtype according to the classification of Verhaak et al.[20] were downloaded from the cBio portal. Methylation cluster types of Ceccarelli et al.[21] and Sturm et al.[22] were obtained from the original paper. RNAseq V3 normalized data was downloaded from firebrowse and was available for 107 samples meeting the above-mentioned criteria. 450k array methylation data was downloaded from the TCGA data portal (https://portal.gdc.cancer.gov/) and was available for 67 of the above-mentioned tumor samples.

*Chinese Glioma Genome Atlas (CGGA) data analysis*

CGGA mRNA microarray data of diffuse gliomas[23] including clinical information was downloaded from the CGGA website (http://www.cgga.org.cn) on October 25th, 2016. Tumors with *IDH* mutations were excluded. For analysis of short and long surviving patients, only samples of patients with survival times < 6 months and > 12 months of altogether 55 patients were used (table S1).

*mRNA data and ingenuity pathway analysis (IPA)*

RNAseq data from TCGA samples were downloaded from firebrose.org as described above. We only used samples with available RNAseq data and *IDH* wild type as well as determined *MGMT* promoter methylation status (*n* = 107) (Details in the Supplement).

*Gene set enrichment analysis (GSEA)*

Gene set enrichment analysis was performed with RNAseq data from TCGA samples and microarray data from CGGA samples downloaded as described above from firebrose.org and cgga.org.cn (see Supplement).

*Cluster analysis*

Consensus clustering using k-means was performed with the 10,000 most variable methylation positions of the indicated dataset. Clusters were calculated with the R extension package 'ConsensusClusterPlus'[24] version 1.38.0. Euclidean distance was used for distance measure. The maximum number of evaluated clusters were k = 8.

*Statistical analysis and graphics*

Univariate survival analyses were performed using the Kaplan-Meier estimator and the log-rank test using the Sigmaplot 12.5 software (Systat Software, Erkrath,

Germany). The exact Fisher-Test was used for significance testing for experiments with a 2x2 matrix. Values of $p < 0.05$ were considered significant and asterisked. Multiple testing was corrected using the Benjamini-Hochberg procedure. Circos plots were generated using the R version 3.3.2 with the extension package 'OmicCircos'[25]. Heatmaps and all further graphics were generated using the extension packages 'gplots' and 'ggplot2'.

## Results

*Differences in the molecular profile of mMGMT and uMGMT tumors at diagnosis*

There were 435/1028 (42%) *mMGMT* and 593 (58%) *uMGMT* samples. Patients with *mMGMT* promoter in the Heidelberg cohort had a better outcome ($p = 0.001$, figure 1A). In the TCGA cohort, survival analysis of patients who received chemotherapy showed a better outcome in the *mMGMT* tumors subgroup, while no difference in patients not receiving chemotherapy ($p = 0.008$, $p = 0.001$ and $p = 0.94$, respectively, figures 1A and 1B).

No significant differences in *cyclin dependent kinase 4* (*CDK4)*, *mouse double minute human homolog* (*MDM2)*, *cyclin dependent kinase inhibitor 2A* (*CDKN2A)* and *platelet derived growth factor alpha* (*PDGFRA)* were found when stratifying for *MGMT* promoter methylation status (figure S1A).

There were no differences between *mMGMT* and *uMGMT* tumors in the general DNA copy number profile (figure 1C). The percentage of copy number altered genome between *mMGMT* and *uMGMT* tumors did not differ in the TCGA dataset (figure 1D).

In TCGA[26], a higher overall number of mutations were reported for *mMGMT* tumors. The absolute number of mutations was higher in *mMGMT* tumors in in the current TCGA cohort (46 vs. 38, $p < 0.001$, figure 1E). None of the mutation frequencies of any single gene differed significantly between *mMGMT* and *uMGMT* tumors, but there was a trend towards a higher frequency of *PTEN* mutations in the *mMGMT* group (31.4% vs. 18.5%, $p = 0.071$). *TERT* promoter mutations BRAF mutations were analyzed in a large dataset by Arita el al.[27] After exclusion of *IDH* mutant and lower grade tumors no differences in *TERT* promoter mutations and *BRAF* mutation frequencies were found according to *MGMT* promoter methylation (*TERT* 57.9% vs 57.8%, *BRAF* 2.8% vs 2.5%, for *mMGMT* and *uMGMT* respectively).

After excluding CpGs in the proximity to the *MGMT* gene, we found 2024 positions (DMP) with an adjusted *p*-value ≤ 0.001 and 438 regions (DMR) differentially methylated between *mMGMT* and *uMGMT* tumors in our 450k array datsets TCGA (figure 3A). Filtering by including only CpGs within the promoter region of a gene identified 814 differentially methylated positions in 419 different genes (table S2). All but three of these positions were hypermethylated in *mMGMT* tumors. Gene set enrichment analysis of differentially methylated positions and regions revealed that the top molecular functions of genes in hypermethylated regions in *mMGMT* tumors involve *transcription regulation and DNA binding* (table S3). Applying a more stringent gene selection, we included genes with at least three differentially methylated CpGs in one DMR. Ingenuity network analysis of the 87 genes matching these criteria found the highest scoring network associated with the functions *cell death and survival* (figure S1B).

*Gastrulation brain homeobox* (*GBX*) 2, a transcription factor known to stimulate proliferation of prostate cancer cells[28], was hypermethylated at five CpGs in the transcription start site of the methylation dataset (adj. *p*-values = $3.7 \times 10^{-6}$ – $6.5 \times 10^{-7}$, figure S2A) in the *mMGMT* group and furthermore more than two-fold downregulated on RNA level in the *mMGMT* group in the TCGA dataset, suggesting transcriptional repression through promoter methylation in *mMGMT* tumors (figure 2). Direct comparison in 40 samples from TCGA with methylation and RNAseq data revealed relevant *GBX2* expression only in samples with low methylation of *GBX2* and these tumors were predominantly *uMGMT* (figure S2B). In addition to *GBX2*, 16 genes were hypermethylated at a minimum of three CpGs in the combined methylation analysis and downregulated in the TCGA RNAseq dataset (figure 2, table S2).

Consensus clustering using k-means of all samples revealed three different subgroups (figure 3A-D). *mMGMT* tumors were overrepresented in cluster 1 (44% vs. 31%, $p = 0.0001$) and underrepresented in cluster 3 (28% vs. 40%, $p = 0.0001$, figure 3E). Comparison of tumors from TCGA included in the analyses of Cecarelli et al.[21] and Sturm et al.[22] revealed that cluster 1 mainly contains the “LGm4” tumors of Cecarelli et al. [21] and the “RTKII classic” tumors of the Sturm et al. classification [22] (figure 3F). Tumor samples in cluster 1 showed higher mean methylation beta-values of differentially methylated CpGs between *mMGMT* and *uMGMT* tumors than tumors in clusters 2 and 3 (figures 3G), $p = 2 \times 10^{-92}$ and $p = 2 \times 10^{-186}$). A hotspot of differentially methylated regions on chromosome 6p21-6p22 is described in the Supplement. Age at diagnosis was similar between the three clusters (cluster 1: 65.5 years, cluster 2: 62.7 years, cluster 3: 61.7 years, p values: cluster 1 vs. 2: 0.09, cluster 1 vs. 3: 0.05, cluster 2 vs. 3: 0.65). The survival difference between patients

with *mMGMT* and *uMGMT* tumors were largest in patients with tumors of cluster 1 (figure S4A, $p < 0.001$ for cluster 1) and no significant survival difference according to *MGMT* promoter methylation was found for clusters 2 and 3. Chemotherapy was given more often in patients of cluster 1, but survival curves were similar when only including patients that received chemotherapy (figure S4B). Overall, patients of cluster 2 showed worse survival (figure S4C). Of note, Nguyen et al.[29] suggested a survival benefit of patients with *mMGMT* only in *TERT* promoter mutated tumors. As *TERT* promoter mutation increases TERT expression, we analyzed the tumors in the different clusters with available RNAseq data. Tumors of cluster 1 showed a considerably higher TERT expression levels than tumors of clusters 2 and 3 (figure 3H, *p* values: cluster 1 vs. 2: 0.021, cluster 1 vs. 3: 0.010). Conversely, the tumor necrosis factor (TNF) - nuclear factor (NF)κB score was highly enriched in tumors of cluster 3 (Figure 3I, *p* values: cluster 1 vs. 3: 0.007, cluster 2 vs. 3: 0.002).

*MGMT* showed the lowest *p*-value for downregulation of all recorded genes in the TCGA dataset ($p = 3.9 \times 10^{-13}$, fold change 0.38 for methylated vs. unmethylated). Applying *p* value for difference of $p < 0.05$ and a fold change of > 2 by RNAseq analysis, there were 175 differentially regulated genes.

*Analysis of paired tumor samples*

Twenty (48%) of paired tumor samples showed *MGMT* promoter methylation, whereas 29 (52%) did not (figure 4A). Only samples with the same *MGMT* promoter methylation status at diagnosis and progression were included for paired analysis. Chemoradiotherapy was received by 42/49 (86%) patients, 3 patients were treated

with chemotherapy alone and 4 received radiotherapy alone (table S4). Three of the 20 (15%) *mMGMT* tumors acquired *PDGFRA* amplification or gain at progression, whereas none of the *uMGMT* tumors did. Instead, three progressive *uMGMT* tumors lost their *PDGFRA* amplification (figures 4A-B). The *uMGMT* tumor 19 is shown as an example for an acquired loss of *PDGFRA* (figure 4C upper panel) and an acquired gain of *CDKN2A* gene dosage at progression that occurred frequently only in *uMGMT* tumors (21% vs. 0%, $p$ = 0.031, figures 4A-B). Loss of the of *CDN2A* deletion in *uMGMT* tumor 16 was accompanied by acquired *CDK4* amplification upon progression (figure 4C middle panel). *mMGMT* tumor 15 is an example of loss of *CDKN2A* upon progression paralleled by loss of preexisting *CDK4* and *MDM2* amplifications (figure 4C lower panel). No major differences were observed in the global CNV profiles of paired tumor samples according to *MGMT* promoter methylation status (figures S5A-B).

*The frequency of PDGFRA, CDK4 and MDM2 amplification in progressive glioblastomas differs according to MGMT promoter methylation*

In 85 samples of progressive glioblastoma (including 66 samples from the Heidelberg database, 13 samples from TCGA patients and 6 samples from Wang et al. [16] CNV of *CDK4, MDM2, PDGFRA* and *CDKN2A* was assessed (figure 5A). Of these tumors, 32 (38%) were *MGMT* promoter methylated and 53 (62%) were unmethylated. Amplification or gain of the *PDGFRA* gene was found in 22% of the *mMGMT* tumors and 7.5% of *uMGMT* samples among progressive tumors ($p$ = 0.092, figure 5B, right panel). Furthermore, amplification or gain of *CDK4* and *MDM2* were found more frequently in progressive *uMGMT* tumors compared to *mMGMT* tumors (3.1% vs.

18.9%, $p = 0.046$ and 0% vs 13.2%, $p = 0.042$ respectively, figure 5B, left and middle panel). When compared to newly diagnosed glioblastomas, the frequency of *CDK4* or *MDM2* amplifications were significantly lower in *mMGMT* tumors in the progressive setting, but were comparable in *uMGMT* tumors (figure S6). In progressive tumors with 450k array data available, we analyzed a set of 19 frequently altered genes in glioblastoma. Besides the differences in *CDK4*, *MDM2* and *PDGFRA* amplifications described above, we found a gain or amplification of the *C19MC* miRNA cluster and the *GLI2* gene more often in *mMGMT* compared to *uMGMT* tumors (54% vs. 22% and 14% vs. 0%, respectively). A higher percentage of *MYB* deletions was detected in *uMGMT* tumors (36% vs. 9%, figure 5C). The higher rate of *C19MC* amplifications in the *mMGMT* group was accompanied by a trend towards a higher amplification rate of the whole chromosome 19 (figure 5D).

PCA of progressive tumors in the 450k methylation array dataset did not separate tumors according to *MGMT* status (figure S7A). Applying the same clustering algorithm as we did for primary tumors, we identified three different methylation groups. *mMGMT* tumors were overrepresented in cluster 1 (57% vs. 20%, $p = 0.005$) and underrepresented in cluster 3 (10% vs. 43%, $p = 0{,}009$, figures S7B-E).

We found 346 DMPs and 182 DMRs between progressive *mMGMT* and *uMGMT* tumors (figure S7F). After filtering for positions within the promoter region of a gene we identified 196 positions in 152 genes that were differentially regulated between both groups. These positions were all hypermethylated in *mMGMT* tumors. Gene set enrichment analysis did not show significantly enriched molecular functions in both groups.

Twenty-one of the 152 genes (14%) had at least one differentially methylated position in the dataset of primary tumors. Of these, within the promoter region of the *myosin light chain (MYL) 12A,* which may be involved in DNA damage repair[30], we identified five positions that were hypermethylated in progressive *mMGMT* tumors (figure S2C). Three of exactly these five positions were already found to be hypermethylated in the dataset of primary tumors.

*Comparison of patients with short and long survival revealed differentially regulated pathways*

We created groups within the TCGA dataset containing samples of patients who survived for either less than 6 months (*mMGMT: n* = 31, *uMGMT: n* =34) or more than 12 months (*mMGMT: n* = 43, *uMGMT: n* = 44) after primary diagnosis. Within both the *mMGMT* and *uMGMT* groups, patients with a short survival time less frequently received alkylating chemotherapy (57% vs. 88% in the methylated group and 47% vs. 86% in the unmethylated group) and were older at diagnosis (67.9 vs. 56.8 years in the methylated and 65.7 vs. 60.0 years in the unmethylated group, table S5). Amplification of the *EGFR* gene was less frequent in tumors with a methylated *MGMT* promoter in patients with death within 6 months after initial diagnosis compared to those who lived longer than 12 months or had an unmethylated *MGMT* promoter or both (29% vs. 50%, $p$ = 0.0048, figure 6A). This corresponded to a reduced mRNA expression of *EGFR* in u*MGMT* patients with short survival in two independent datasets of the TCGA cohort derived from RNAseq and microarray data.

*TP53* mutations were common in *MGMT* promoter unmethylated tumors and in methylated tumors with a short survival, but were significantly less common in *mMGMT* tumors and a survival time of more than one year compared to the group of patients with short survival of less than 6 months (30% vs. 4.5%, $p = 0.041$, figure 6B). Like the lower amplification rate of *EGFR*, there was a trend towards a lower *EGFR* mutation frequency in the *mMGMT* patients with short survival.

RNAseq data of tumors of the groups was available for 50 patients. IPA of differentially regulated genes on RNA level (figure 6C, table S6) revealed a strong prediction of upregulation of TNF-α and the NFκB-complex in tumors from patients with short survival with *mMGMT* compared to patients with longer survival. To confirm this finding, we conducted a GSE analysis with a set of genes for signaling of TNF-α through NFκB provided by the Broad Institute. This gene set was highly enriched in patients with *mMGMT* tumors and short survival (figure 6C, table S7). Conclusively, cellular movement and proliferation were enhanced in short surviving *mMGMT* tumors in the IPA analysis (figure 6E). Further evidence comes from the CGGA cohort, where in *mMGMT* tumors signaling of TNF-α through NFκB was enriched in a patient cohort with worse prognosis[31]. Exceptional high TNF-NFκB scores were seen in 3/13 (23%) tumors of patients surviving < 6 months. 9/13 (62%) of tumors of the < 6 months group and 3/14 (21%) of tumors of the > 12 months group had scores > 0 for NFκB target genes (suppl. figure 9A-B). Additionally, the same pathway was enriched in the dataset of tumors from the TCGA collective with available microarray data (figure S8A).

Signaling through interferon-α was the only pathway strongly activated in the group of tumors from patients with longer survival (figure 6D, table S6) translating into a

decreased function “infectious disease” (figure 6E), which has been confirmed by GSE and CGGA as response to interferon-α was the top enriched in patients with high *MGMT* gene expression and survival times of more than 12 months (figure S8B). *STAT3* was both predicted to be upregulated in *mMGMT* and *uMGMT* tumors of patients with short survival compared to patients with longer survival times (figures 6C and 6D).

## Discussion

*M*ost of the *IDH* mutant glioblastomas are *MGMT* promoter methylated[32]. In this study, genetic, epigenetic and transcriptional differences between *MGMT* methylated and unmethylated IDH wldtype glioblastomas have been explored.

CNV and mutations do not differ between newly diagnosed *mMGMT* and *uMGMT* tumors. Survival data of patients from the TCGA cohort who received sole radiotherapy showed no difference in overall survival of *mMGMT* and *uMGMT* patients making it likely that minor changes in the molecular profile do not affect the clinical course of the disease in the absence of temozolomide.

Methylation analysis with consensus clustering of more than 1000 glioblastoma samples revealed three different methylation clusters sharing similarities with published work[21,22]. The allocation into three clusters is relatively stable between different datasets. We found that *mMGMT* tumors were enriched in cluster 1 and *uMGMT* tumors in cluster 3. Cluster 1 shows higher average methylation values, explaining why almost all differentially methylated CpGs between *mMGMT* and *uMGMT* tumors are hypermethylated in *mMGMT* tumors. There are no distinct

methylation patterns of *mMGMT* and *uMGMT*, but *MGMT* promoter methylation is unevenly distributed within three main methylation clusters, with a higher chance of having a methylated *MGMT* promoter in the cluster with the highest average methylation. The survival advantage of *MGMT* promoter methylation was only present in the more favorable cluster 1, supporting that the prognostic impact of MGMT is dependent on the global methylation profile. Tumors of cluster 1 express higher levels of TERT, suggesting *TERT* promoter mutations being more frequently in that cluster where survival was strongly associated with *MGMT* promoter methylation. Patients with tumors of clusters 2 and 3 have low TERT expression and do not show a survival benefit with *mMGMT*, confirming *mMGMT* is only a survival advantage in high TERT expressing tumors[29]. Furthermore, TERT expression/promoter mutations might be associated with specific epigenetic subgroups with high methylation levels and the enriched high TNF-NFκB scores in cluster 3 may partly explain the chemoresistance in this cluster. However, these findings should be validated in further studies.

A hypermutated genotype in some *MGMT* promoter methylated tumors upon progression had been published[8]. We specifically analyzed CNV and found *PDGFRA* predominantly amplified in *MGMT* promoter methylated progressive tumors. In paired samples, we found that *uMGMT* tumors loose *PDGFRA* amplifications upon progression, whereas *mMGMT* tumors tend to gain amplifications of *PDGFRA.* This might also be a resistance mechanism to therapy, especially in *mMGMT* tumors that are more vulnerable against chemotherapy with temozolomide.

The frequency of *CDK4* and *MDM2* amplifications tended to be higher in progressive *MGMT* promoter unmethylated tumors, and in such cases preexisting *CDKN2A*

losses may change upon progression to a balanced state, which is consistent with the finding that *CDKN2A* loss and *CDK4* amplification rarely occur in parallel regardless of *MGMT* promoter methylation status[33]. These specific changes cannot be explained by the function of the MGMT protein and are likely to be a response of the tumor to different efficacy of the alkylating chemotherapy. However, the CNV differences of *PDGFRA*, *CDK4* and *MDM2* are based on a limited tumor dataset and should therefore be viewed as preliminary. Robust statistical validation from larger number of samples will be required.

In our comparison of patient groups with shorter versus longer survival we found differences in specific CNV as well as mutation frequencies and different gene expressions according to *MGMT* promoter methylation. However, a potential bias could be due to the lower number of patients receiving alkylating chemotherapy in both the methylated and unmethylated groups of patients who survived for less than 6 months compared to the respective groups of patients living longer than 12 months.

The predicted activation of the TNF-NFκB pathway in the group of short surviving *mMGMT* patients is supported by previous preclinical data showing upregulation of MGMT through high NFκB signaling and therefore increased chemoresistance [34]although this may not be uniformly dependent on the level of promoter inactivity, possibly contributing to the short survival times in patient with otherwise favorable *MGMT* promoter methylated tumors. Of note, it is more likely that glioblastomas of patients with longer survival show a downregulation of TNF-NFκB signaling, as we also found a higher NFκB signaling in unmethylated tumors not selected for survival compared to all methylated tumors and no difference when comparing tumors from

patients with short survival and *MGMT* promoter methylated tumors against all patients with unmethylated glioblastomas.

Likewise, interferon signaling through interferon α/β can sensitize *MGMT* promoter unmethylated glioblastomas to temozolomide[35,36]. There is an upregulation of the interferon-α pathway in *uMGMT* tumors and long survival compared to both patients with unmethylated tumors and short survival as well as patients with methylated tumors and long survival, suggesting that patients who survive relatively longer with the unfavorable *uMGMT* might have this survival benefit from increased interferon-α signaling and therefore increased chemotherapy sensitivity. This is of particular importance because most of the patients in the group of patients with longer survival despite having tumors with *uMGMT* received temozolomide. The data is supported by a dataset of the CGGA, although caution is required because of the low sample size. The predicted upregulation observed in our study supports the recently proposed therapeutic inhibition of STAT3 in glioblastoma patients[37], at least for patients with glioblastomas lacking *MGMT* promoter methylation.

In conclusion, *MGMT* promoter methylation status serves as a biomarker for temozolomide therapy and does not determine an otherwise molecularly distinct glioblastoma subpopulation in the newly diagnosed setting. The chance to find a molecular target for trial inclusion is not *per se* worse in the *MGMT* unmethylated population. Further, there is a clear signal from this data to demand a new tissue analysis for every trial at recurrence. Lastly, the data provide evidence for the TNF/NFκB pathway to be revisited in future trials. Temozolomide might be safely omitted in patients with *MGMT* promoter unmethylated glioblastomas in clinical trials,

especially when meaningful molecular markers predicting the response to experimental therapies are found by predictive molecular analyses.

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## Figure legends

### Figure 1: Copy number alterations in primary glioblastoma according to *MGMT* promoter methylation

**(A)** Left panel: Overall survival of 143 glioblastoma patients with *IDH1* wildtype tumors according to *MGMT* promoter methylation. Right panel: Survival analysis of patients with tumors of the TCGA dataset regardless of the treatment. **(B)** Left panel: Survival analysis of tumors of the TCGA glioblastoma database not treated with chemotherapy according to *MGMT* promoter methylation. Right panel: Survival analysis of the TCGA dataset treated with chemotherapy. **(C)** Graphical illustration of the percentage of CNV in the *mMGMT* and *uMGMT* group. **(D)** Relative fractions of DNA copy number alterations detected in the TCGA database according to *MGMT* promoter methylation. Shown are the means ± standard deviation. **(E)** Average fraction of mutations detected in the TCGA database according to *MGMT* promoter methylation. Shown are the means ± standard deviation.

### Figure 2: Differential methylation in primary glioblastoma according to *MGMT* promoter methylation

From outside to inside of the circle: 1st circle: Hypermethylated genes in *mMGMT* tumors according to their chromosome position. Genes that were additionally downregulated in the RNAseq TCGA dataset are labeled in black, others in light red. 2nd circle: All differentially methylated positions within a promoter region of a gene. 3rd circle: Area of each DMR between *mMGMT* and *uMGMT* glioblastomas. 4th circle: All differentially methylated positions, including MGMT related on chromosome

10. Center: GBX2 mRNA expression in the TCGA dataset in *mMGMT* and *uMGMT* glioblastomas.

**Figure 3: IDH wildtype glioblastoma cluster into three different clusters**

**(A)** Heatmaps of 2024 positions that are differentially methylated between *mMGMT* and *uMGMT* tumors in the dataset of primary tumors, excluding *MGMT* related and intergenomic positions. **(B)** Consensus clustering using k-means of all samples of the primary tumor dataset. Shown is the result for k = 3. **(C)** Plot showing the relative change in area under the CDF curve in the k-means unsupervised clustering of primary glioblastoma. **(D)** CDF plot with examples from k = 2 to k = 8. Three clusters were chosen as the optimal number**. (E)** Distribution of *mMGMT* and *uMGMT* primary tumors into the clusters 1, 2 and 3. **(F)** Comparison of the cluster assignments with the clusters from Cecarelli et al. [21] and Sturm et al. [22] for 65 glioblastoma samples of the TCGA database. **(G)** Mean methylation beta-values of the 2024 differentially methylated CpGs, **(H)** relative TERT expression and **(I)** TNF-NFκB score according to methylation clusters. The lower and the upper hinges correspond to the first and third quartiles. The upper and lower end of the whiskers correspond to the 1.5x IQR (inter quartile range) from the hinge.

**Figure 4: CNV changes in pairs of primary and progressive glioblastoma**

**(A)** CNV of the genes *CDK4, CDKN2A, MDM2* and *PDGFRA* in a set of 49 paired glioblastoma samples at primary diagnosis and progression. *MGMT* promoter methylation is indicated in the first row. **(B)** Shift in gene dosages of *CDK4*, *CDKN2A*,

*MDM2* and *PDGFRA* in paired samples of primary and progressive glioblastoma according to *MGMT* promoter methylation. **(C)** Examples of copy number profiles of three paired primary and progressive glioblastoma samples. Color code: *PDGFRA* = red, *CDKN2A* = yellow, *CDK4* = blue, *MDM2* = green.

**Figure 5: Molecular differences between *uMGMT* and *m*MGMT progressive glioblastomas**

**(A)** CNV of *CDK4*, *MDM2*, *PDGFRA* and *CDKN2A* in the cohort of the progressive tumors, grouped according to MGMT promoter methylation. **(B)** Comparison of *CDK4*, *MDM2* and *PDGFRA* copy number variations between *mMGMT* and *uMGMT* tumors in the progressive situation. **(C)** Heat map of CNV of at set of 19 frequently altered genes in the progressive tumor collective. High frequencies of alterations are shown in red, low frequencies in blue. *MGMT* promoter methylation is indicated in the first row. **(D)** Copy number variation profiles in *mMGMT* and *uMGMT* tumors in the progressive situation.

**Figure 6: Comparison between glioblastomas of patients with different survival (< 6 months vs. > 12 months) according to *MGMT* promoter methylation status**

**(A)** Copy number variations of *CDK4, EGFR* and *PDGFRA* according to *MGMT* promoter methylation status and survival groups (< 6 months vs. > 12 months) of glioblastoma patients in the TCGA cohort. **(B)** Mutations of *EGFR* and TP53 according to *MGMT* promoter methylation status and survival groups of TCGA glioblastoma patients. **(C)** GSE analysis (left) and IPA network analysis (right)

comparing results obtained for *mMGMT* tumors of patients with survival times < 6 months vs. > 12 months. Prediction for upregulation is indicated in orange. **(D)** GSE analysis (left) and IPA network analysis (right) comparing results obtained for *uMGMT* tumors of patients with survival times < 6 months vs. > 12 months. Prediction for upregulation is indicated in orange, for downregulation in blue. **(E)** IPA of patients with survival times < 6 months vs. > 12 months. Enriched differentially regulated functions are visualized and the topics cellular movement, cell survival, signaling and proliferation as well as infectious disease are highlighted in red.

**Figure 1**

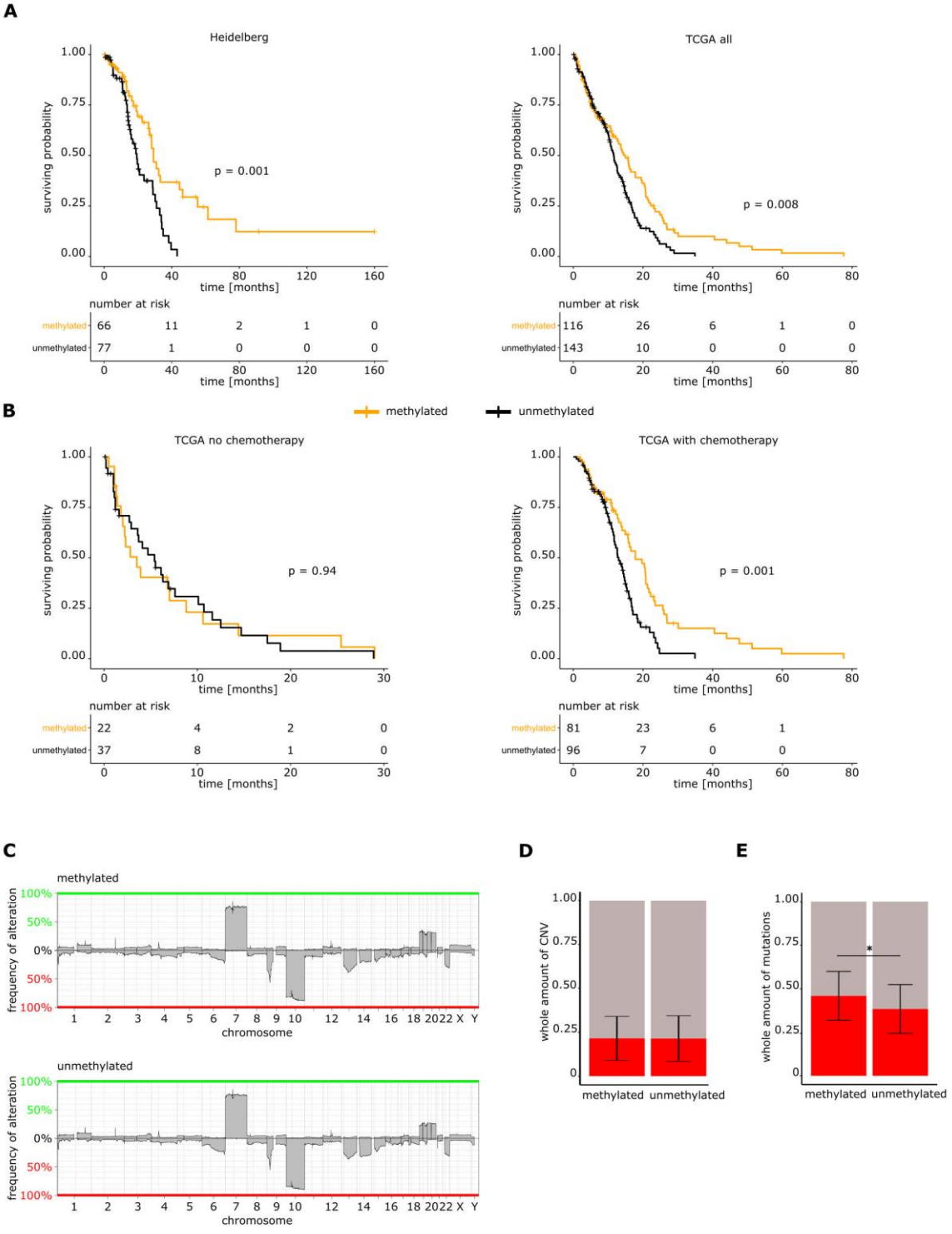
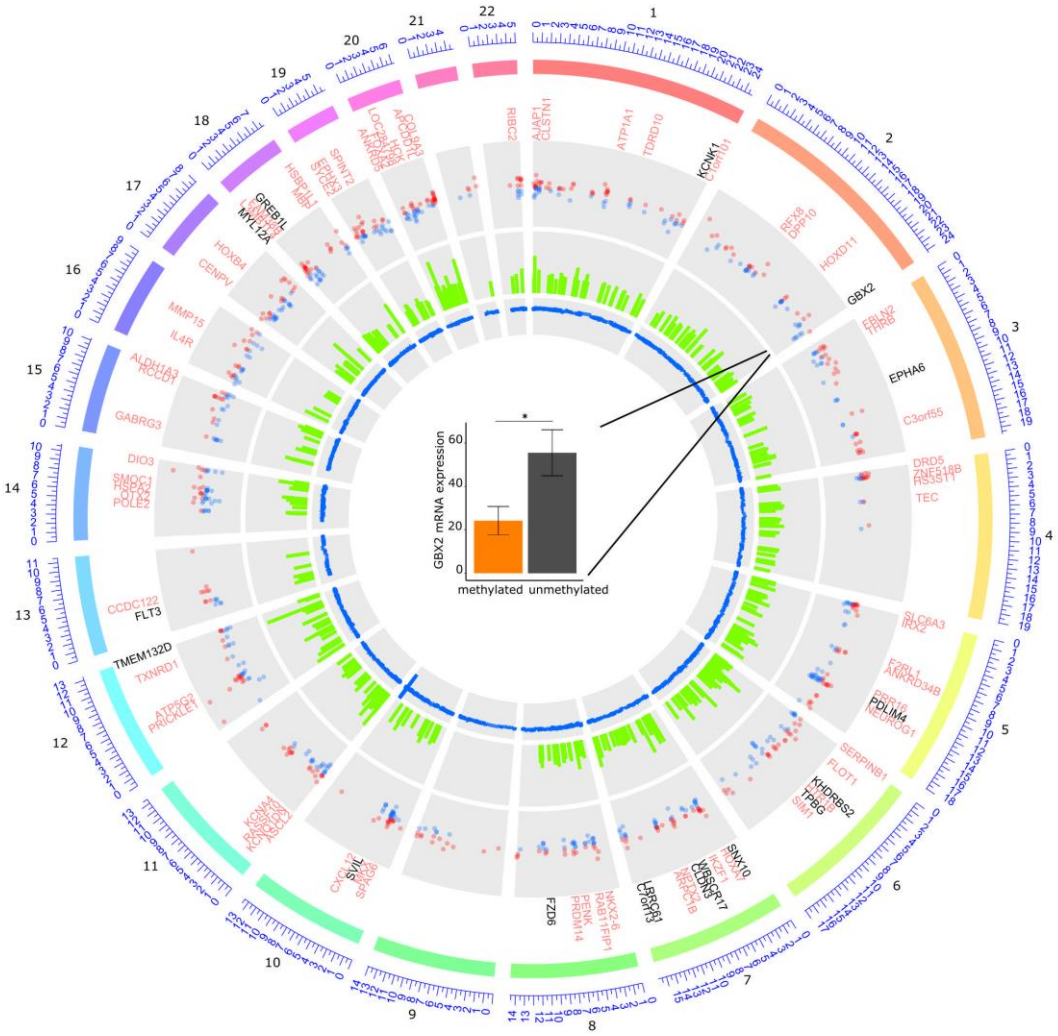


**Figure 2**



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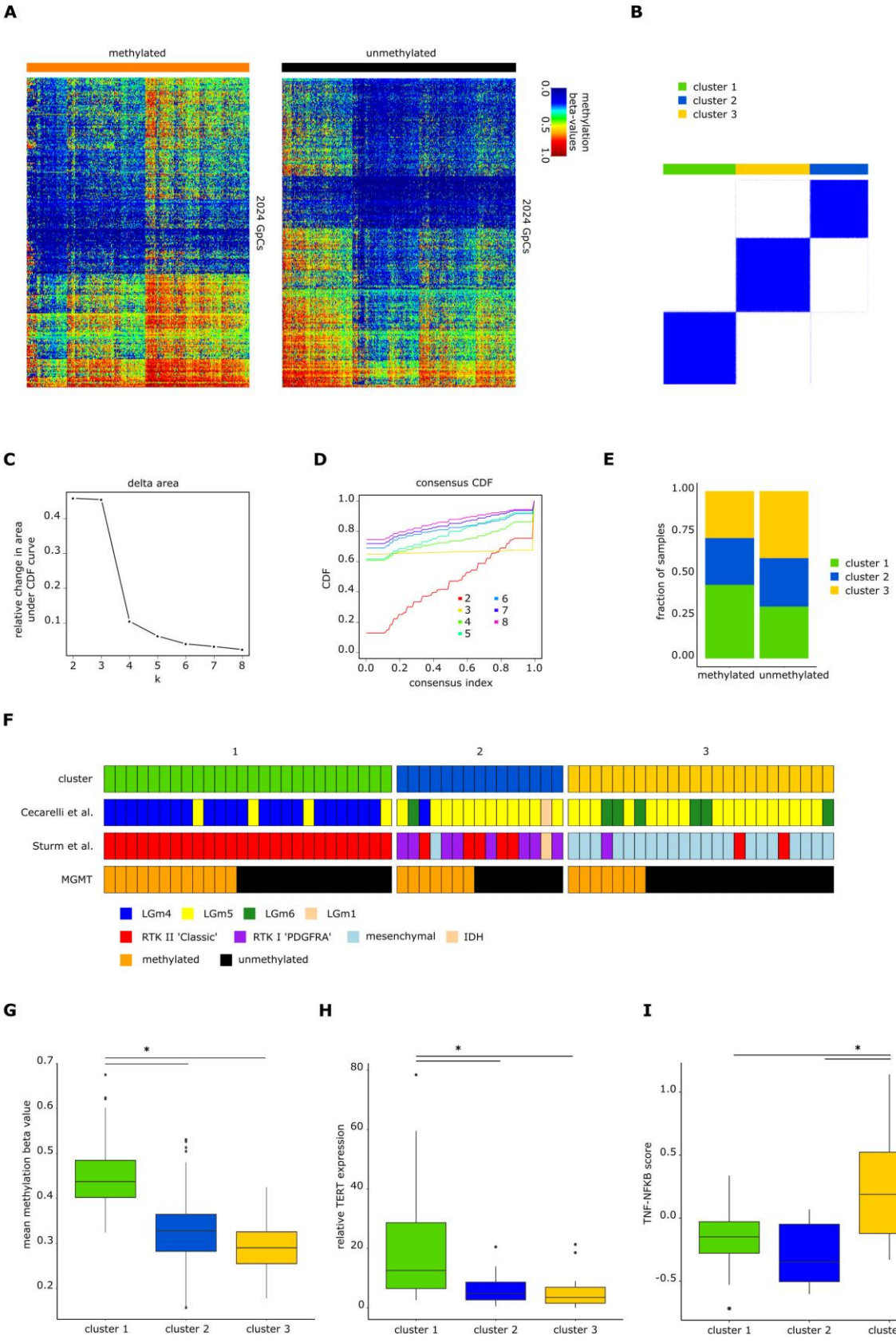

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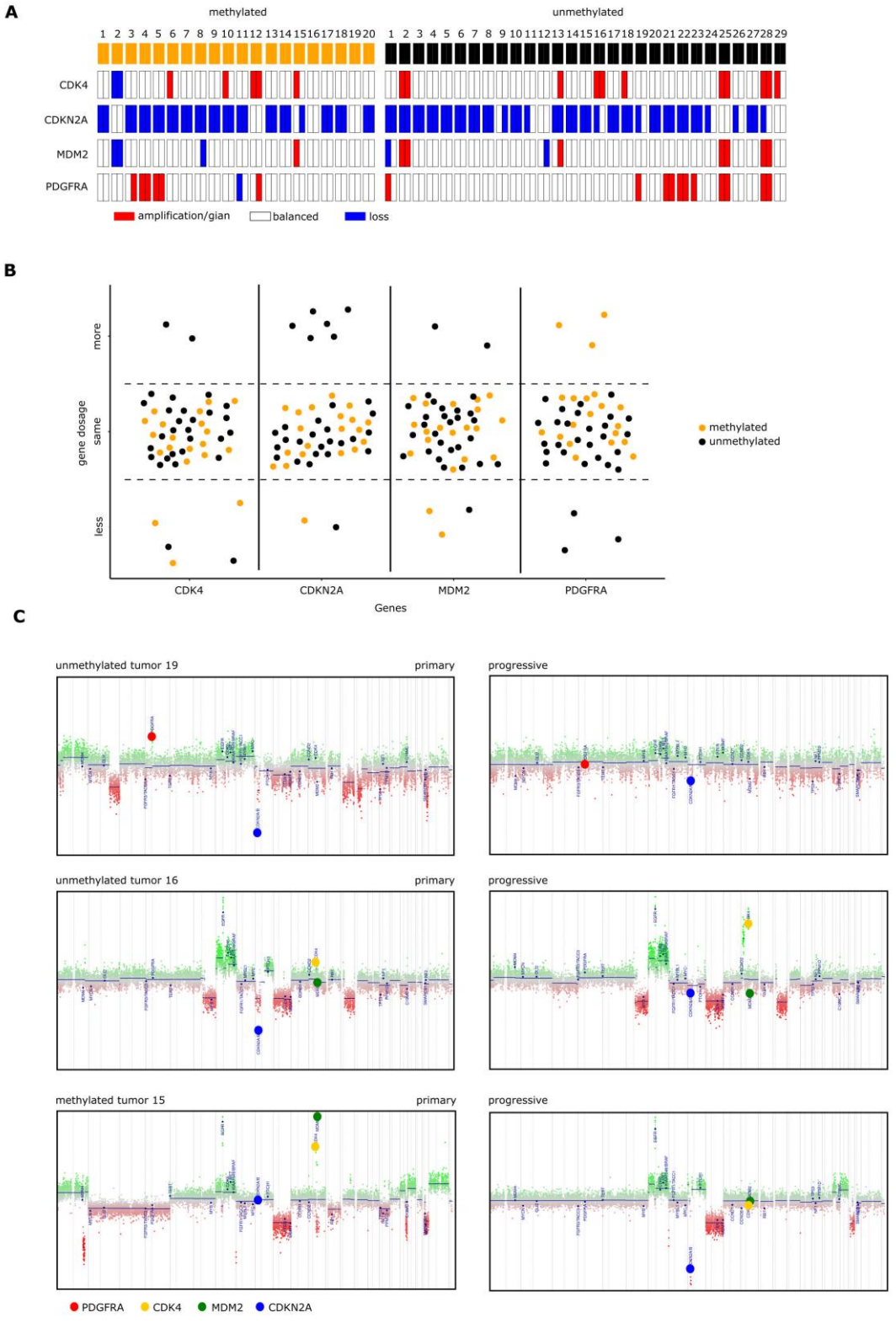

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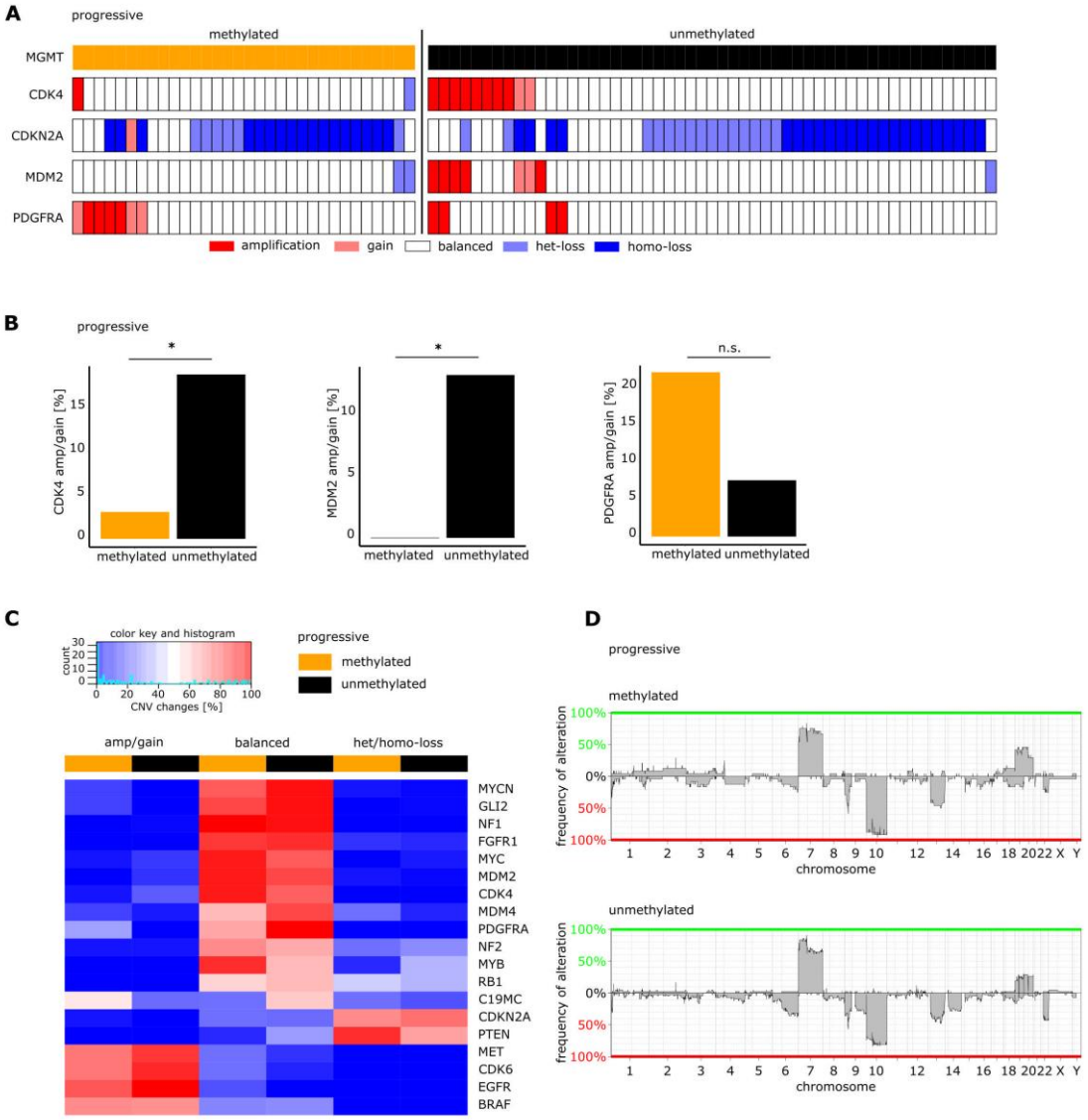

**Figure 6**